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INVESTIGATION ON MUSCLE PROTEIN DEGRADATION AND ITS ROLE IN PALE, SOFT, EXUDATIVE (PSE) PORK

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INVESTIGATIONS ON MUSCLE PROTEIN DEGRADATION AND ITS ROLE IN PALE, SOFT, EXUDATIVE (PSE) PORK

ADF Project # 20000224

Final Report

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EXECUTIVE SUMMARY

The Saskatchewan meat industry is continuing to expand pork production and markets for both domestic and international customers and must strive to offer consumers products of the highest quality. A number of antemortem and postmortem factors can significantly impact pork meat quality. For example, when pigs have an abnormally rapid rate of postmortem glycolysis, causing a rapid pH decline, the resulting raw meat is often pale in color, soft in texture and exudative (PSE). Not only is eating quality of the fresh meat affected, but processing quality can be severely compromised. The current incidence of PSE is reported to be at 15.5% resulting in an estimated loss in value of $0.90 USD per carcass (Stetzer and McKeith, 2003). In their survey of the US pork supply chain, PSE was the third highest contributor (after inconsistent weights and thin bellies) to product inconsistencies reported by packers.

This initiative offered a unique interdisciplinary and integrated approach to study factors contributing to the poor processing quality of PSE pork meat by combining assessments of fresh and processed meat quality with physicochemical analyses, biochemical properties and immunocytochemical data at the cellular level. The objectives of this study were: (1) to study the contribution that protein denaturation and degradation makes to texture and water holding in meat gels, (2) to delineate the cause and mechanism(s) of protein degradation of myosin and other muscle proteins in PSE pork, (3) to evaluate the effect of fiber type on development of PSE and on protein degradation, and (4) to identify possible control strategies to limit protein degradation and gelation impairment in PSE muscle.

Pork samples were selected from a commercial processing line (through the cooperation of Mitchell's Gourmet Foods) on several occasions and were classified as either normal or PSE (pale, soft, exudative) based on pH, color and water holding capacity. Following evaluation of the properties of the fresh meat, samples were collected for biochemical and immunocytochemical evaluations with some meat processed into low-fat meat batters.

Protein denaturation of muscle proteins from PSE meat was confirmed by the increased surface hydrophobicity compared to that in normal meat. This change in protein conformation is likely due to alteration of the tertiary and/or quaternary structures of the muscle proteins rather than their secondary structures since the α-helical contents of actomyosin from normal and PSE meat were similar. The observation that surface hydrophobicity of muscle proteins from PSE meat is significantly different that that of normal meat may be of value to meat scientists as a basis for developing a tool for rapidly distinguishing meat quality.

That muscle proteins from PSE meat is not only denatured but also degraded was revealed by Western blotting analysis using antibodies against myosin. The cross-reactivity of 9 commercial myosin antibodies was evaluated for sensitivity and selectivity. Two antibodies were found useful in the assessment of degradation since they cross-reacted with large fragments of myosin. Our study showed that myosin from PSE meat is more degraded that myosin in normal meat.

Furthermore, storage of normal meat at 4 °C resulted in progressive degradation with time of storage.

The low pH of PSE meat may have disrupted chemical interactions that maintain the conformation of a muscle protein such as hydrogen and ionic bonds, resulting in a conformation that is susceptible to proteolysis. In an attempt to identify the protease(s) that is responsible for the degradation of myosin from PSE meat, digestion patterns of myosin by proteases (calpain, cathepsin B, cathepsin D), which are likely to be active at acidic pH, were compared to those of muscle proteins from PSE meat and stored normal meat. All three enzymes proteolyzed myosin and yielded different digestion patterns. Our comparative study showed that the degradation of myosin in PSE meat is a consequence of the activity of more than one protease. Furthermore, significant inhibition of proteolysis was observed when the pH of the meat was increased to 8.0.

A contributing factor to the enhanced acidic condition in PSE is the glycolytic activity of the anaerobic muscle fiber type 2b. Comparison of the muscle fiber types of normal and PSE meat revealed that the relative contribution of each muscle fiber type (1, 2a, 2x or 2b) to the cross-sectional area of the muscle did not vary between PSE and normal (control) loins. The lack of differences in fiber types between PSE and control animals indicates that factors other than fiber type alone must be responsible for the acquisition of the PSE condition. The smaller fiber size observed for meat from PSE animals is consistent with loss of fluid from the muscles of these animals.

The meat gels prepared from PSE pork had significantly lower textural quality as measured by torsional gelometry (lower shear stress and shear strain at failure) and texture profile analysis (lower hardness) and lower water holding capacity as shown by higher cooking loss, expressible moisture and purge than comparable products prepared from normal meat. Differential scanning calorimetry suggested that the inferior quality of meat gels from PSE meat might have resulted from their lower protein thermal stability.

The comprehensive studies of thermally-induced gelation of natural actomyosin (NAM) from normal and PSE pork carried out during this project allowed us to postulate a more detailed mechanism of meat protein gelation. The different thermally-induced gelation behaviour of NAM extracted from normal and PSE pork was attributed to the denaturation status of the original proteins. NAM from normal pork underwent aggregation through a higher extent of hydrophobic interaction and disulfide bonds, as well as higher temperature in folding and maximum S0-ANS change than that from PSE pork. As a consequence, NAM from normal pork had superior rheological properties. Further study of PSE development in the early post mortem period may hold further clues regarding quality and conformation of the major proteins in pork.

This work provides new insight into how the structure of meat proteins (from both normal and PSE meat) is changed (denaturation and degradation) during refrigerated storage, frozen storage and thermally-induced gelation. Therefore, in addition to the consideration given for genetic

improvement of meat quality, preventing meat protein denaturation, in particular, immediately after pork slaughter should be an important consideration for the meat industry.

SHORT ABSTRACT

Protein changes (denaturation and degradation) in normal and PSE (pale, soft, exudative) pork were evaluated in an interdisciplinary study involving fresh meat quality, meat processing, and physicochemical, biochemical and immunocytochemical analyses. We determined that physicochemical properties of proteins from PSE meat were markedly different than from normal muscle. In particular, surface hydrophobicity of actomyosin was higher when extracted from PSE meat, providing new information on denaturation. With frozen storage, hydrophobicity increased further. Evidence for protein degradation in PSE meat as well as storage of normal meat at 4 °C was also demonstrated by western blotting analysis of the muscle proteins. No one protease was determined to be responsible, nor did fiber type influence predisposition to PSE. However, alkaline pH inhibited proteolysis confirming that the acidic PSE condition promoted protein degradation. Our evaluation of gelation events allowed us to postulate a detailed sequence of events involved in gelation of fibrous proteins such as myosin, and to closely document the effect of PSE on gelation. This research has furthered our understanding of the relationship between post mortem events and resulting meat quality and gelation.

INTRODUCTION

One of the greatest challenges confronting the pork manufacturing industry today is the demand for high quality meat products. Pale, soft, exudative (PSE) pork has been documented for more than 50 years. Pork with PSE properties is a significant meat quality problem causing multi-million dollar financial costs in the pork industry (Frisby *et al.*, 2005). According to surveys, the incidence of PSE pork in the United States of America was 18% in 1963, 16% in 1992 (Cassens, 2000) and 15.5% in 2001 (Stetzer and McKeith, 2003). In 2002, it was estimated that U.S. pork industry loses $0.90 US dollar per carcass due to PSE (Stetzer and McKeith, 2003). Since about 30 million hogs were produced annually in Canada, this would translate into more than $30 million Canadian dollars of losses to the Canadian pork industry. Studies from the live animal to highly controlled *in vitro* studies indicate that genetics, nutrition and management, pre-slaughter animal handling, stunning, dehairing and carcass chilling can influence the incidence and magnitude of the PSE condition (Cassens, 2000). Various strategies such as gene testing, good pre-slaughter handling, quick chilling, and carbon dioxide stunning are being employed to minimize the incidence of PSE pork. Although pigs with a genetic predisposition have been eliminated from production since the early 1990s, the PSE problem still persists. Thus, PSE remains an important area of research.

It has been generally accepted that the development of PSE results from the increased glycolysis rate post mortem. The rapid decline of pH in PSE muscle, combined with higher carcass temperature within the first few hours post mortem, cause the proteins in the muscle to denature. For food scientists, denaturation of protein is of great importance since it has such a large influence on colour, texture and protein functionality, *e.g.*, water holding capacity, viscosity, emulsion stability, protein solubility and gelation. From a biochemical point of view, denaturation of a protein involves structural changes in the secondary, tertiary or/and quaternary structure due to alteration of hydrogen bonds, hydrophobic interactions, ionic linkages or interchange reactions of covalent disulfide bonds. Another important post mortem change in protein is protein degradation. Protein degradation can be defined as the disruption of peptide bonds resulting in protein fragments that may or may not retain biological activity and/or structural function. Compared with the intensive study of protein functional attributes, such as meat water binding capacity, drip loss, cooking loss, cooking purge, protein solubility, emulsion stability, pH and sensory evaluation of meat products, there is little information about the structural properties (*e.g.*, protein hydrophobicity, sulfhydryl groups, alpha-helical content) of proteins from PSE and normal pork. The exact nature of the denaturation of meat protein in PSE muscle has not been explored at the structural level. Knowledge of the cause of degradation and detailed information on the denaturation events would allow us to develop control strategies with the ultimate aim of improving the quality of meat from PSE-prone animals.

The objectives of this project were:

1. to study the contribution that protein degradation and denaturation makes to texture and

water holding in meat gels.
2. to identify if proteolysis by specific proteases (or other mechanisms) is responsible for degradation and impairment of gelation.
3. to evaluate the effect of fiber type on development of PSE and on protein degradation.
4. to develop control strategies to limit gelation impairment in PSE muscle.

SUMMARY OF FINDINGS

Work on this project has been shared between three laboratories, namely Dr. Shand's meat science facilities/pilot plant, Dr. Pato's biochemistry lab and Dr. Rosser's muscle biology lab. This project offers a unique interdisciplinary and integrated approach to the study of PSE by combining meat quality assessments with detailed evaluations of biochemical, immunocytochemical and thermo-rheological properties. Procedures used are summarized in Appendix 1. Activities under each objective are described below.

Objective 1: to study the contribution that protein degradation and denaturation makes to texture and water holding in meat gels.

1.A. Study of protein denaturation and degradation.

It is well known that PSE causes denaturation of protein and impaired meat quality. What is not known is the type of denaturation that results. Denaturation is a consequence of alteration of the secondary, tertiary or quaternary structure of the protein due to disruption of chemical interactions that maintain the protein conformation. The change in conformation could be assessed by physical and biochemical measurements such as circular dichroism and determinations of surface hydrophobicity and sulfhydryl contents. On the other hand, protein degradation is due to proteolysis of peptide bonds resulting in the formation of fragments of the proteins that could be detected by methods such as SDS-polyacrylamide electrophoresis and Western blotting analysis.

1.A.1. Protein denaturation.

Study of denaturation of pork muscle proteins was carried out by comparison of the physical and biochemical properties of natural actomyosin (NAM) prepared from normal and PSE pork. Actomyosin, which accounts for 50-60% of the protein in muscle, is the predominant protein complex in postrigor meat. The biochemical and physical properties of pork NAM were studied as part of the Ph.D. research of Haihong Wang.

The surface hydrophobicity, alpha-helical content, sulfhydryl and disulfide content and apparent viscosity of NAM from normal and PSE pork were monitored and compared during refrigerated storage of NAM, and following frozen storage of pork loin for up to 6 months. The most significant finding was that NAM from PSE animals at 57 hr post mortem had higher surface hydrophobicity than that from normal animals (Figure 1). Interestingly, treatment of meat with sodium nitrite had no effect on hydrophobicity. In addition, refrigerated storage of NAM or frozen storage of the meat tended to further increase hydrophobicity. Reactive sulfhydryl content also changed significantly during refrigerated storage of NAM, but it was not influenced by quality designation. The alpha-helical contents of NAM from PSE and Normal meat were similar.

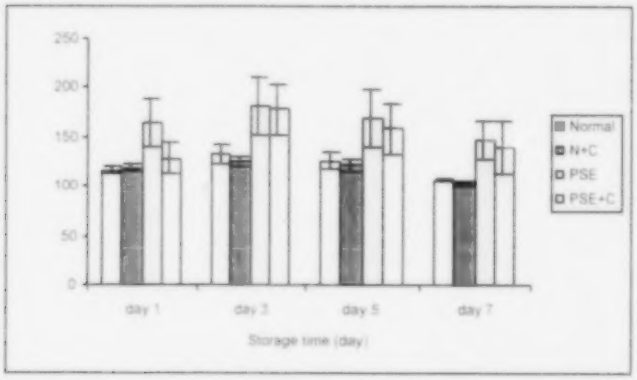

Figure 1. Changes in surface hydrophobicity (S_0) of NAM extracted from normal and PSE pork loin during storage at 4°C as affected by addition of sodium nitrite. White, white with bars, gray and gray with foreground bars show mean values of surface hydrophobicity of normal NAM (Normal), normal NAM with 150 ppm sodium nitrite (N+C), PSE NAM (PSE) and PSE NAM with 150 ppm sodium nitrite (PSE+C), respectively. Vertical bars represent the standard error of the mean, four samples were used for each group.

These studies revealed important new information on surface hydrophobicity (S0-ANS). The S0-ANS of NAM from PSE pork ($p < 0.12$) tended to be higher than that from normal pork. The S0-ANS results suggest that NAM from PSE pork has more exposed aromatic hydrophobic amino acid residues on the protein surface compared with the NAM from normal pork. The higher S0-ANS of NAM from PSE pork provides evidence that denaturation of proteins was more pronounced in PSE pork than normal pork. This conformational change may be subtle, because surface sulfhydryl and alpha-helical content of NAM did not differ between normal and PSE pork.

A promising new methodology could result if the measurement of protein properties can more accurately reflect the meat quality. Thus, the sensitivity of S0-ANS measurement may be valuable to meat science researchers for identification of meat quality. If the measurement procedure for S0-ANS can be simplified, this method could be used by the meat industry to replace conventional methods (combination of measuring pH, drip loss and colour) for identifying meat quality.

1.A.2. Protein degradation

Protein degradation of myosin was studied by SDS (sodium dodecyl sulfate)-polyacrylamide electrophoresis and western blotting analysis using a number of commercial myosin antibodies (Ab 1-9 listed in Appendix 1). The electrophoretic and immunological properties of SDS extracts and washed myofibril (WMF), which is composed mainly of myosin and actin, prepared from normal and PSE pork were compared. Initially, the specificities of these antibodies were determined by their cross-reactivity with purified pork loin myosin from normal and PSE meat, as well as with myosin digests. Purified myosin was digested with calpain, cathepsin B and cathepsin D, enzymes that are likely to be active at conditions at which PSE occurs. These antibodies exhibited different immunological behavior toward myosin. All the antibodies, except for Ab 9 cross-reacted with myosin heavy chain (MHC), the 200 kDa subunit of myosin. It was observed that Ab 5 and 6 bound only to MHC whereas Ab 4 and 8 cross–reacted with the MHC and several other low molecular weight fragments of myosin. Ab 2 and 3 cross-reacted with the MHC and a few low molecular weight proteins. Ab 2 also recognized the 27 kDa myosin light chain. Interestingly, Ab 1 and 7 did not bind to MHC in the normal pork SDS extract and showed weak binding to the purified myosin but it cross–reacted strongly with the MHC in PSE meat. This observation suggests that the epitopes for these antibodies are not easily accessible in the myosin from the normal meat, and become readily available for interaction with the antibodies in PSE meat probably due to degradation or denaturation of the myosin from PSE pork. The lack of cross-reactivity of some myosin antibodies to low molecular weight fragments of MHC does not necessarily mean no protein degradation because their epitopes may be localized in small fragments that were not retained in the gel, and therefore were not detectable by Western blotting. Ab 9 only cross-reacted with turkey myosin, and not with pork and beef myosin.

Having established that Ab 1-8 recognized pork myosin, they were used to show changes in the structure of myosin from normal and PSE meat. Three sets of normal and PSE pork samples collected in 2002, 2003 and 2004 were used for this study. The meat samples were extracted with SDS containing buffer to allow the analysis of detergent soluble proteins. Washed myofibrils (WMF) were prepared from the meat samples to examine the properties of the muscle proteins, particularly myosin. Ab 4 and 8 were most valuable in showing differences between these samples since they cross-react with fragments of MHC. It was observed that these antibodies cross–reacted strongly with the MHC in SDS-extracts and WMF of normal meat. In some cases, weak bands of lower molecular weight (60 - 170 Da) fragments were observed in these samples. On the other hand, the PSE meat samples showed strong MHC band and its degradation products (Figure 2). These results provide evidence for increased degradation of myosin in the PSE samples. Detection of the difference in the cross-reactivity of the normal and PSE samples with the antibodies required careful determination of the appropriate concentrations of the primary and secondary antibodies for use in the assays. For example, this difference was not observed when the primary antibody was diluted 1:1000 but was observed with more dilute antibody solution (1:100,000). Examination of the SDS extracts and WMF from normal and PSE pork on SDS-

polyacrylamide gels, did not reveal any significant difference indicating that the use of the antibodies is a more sensitive method for detecting subtle changes in the structure of the protein.

Our studies also showed that storage of pork at 4 °C results in degradation of myosin. Normal and PSE meat were stored at 4 °C for 2, 5, 24, 57, 77, 99, 123 and147 hours, and their SDS extracts and WMF were analyzed for degradation using various antibodies. In general, it was observed that degradation increased with time in the normal meat (Figure 2). On the other hand, the PSE pork samples showed the presence of significant amounts of degradation products in all the samples analyzed. The PSE patterns resembled that of the normal meat that was stored for 123 hours and longer. These results suggest that the myosin undergoes proteolysis upon storage, and in PSE condition, which is acidic pH. This is consistent with the observed increase of surface hydrophobicity of NAM upon refrigerated storage.

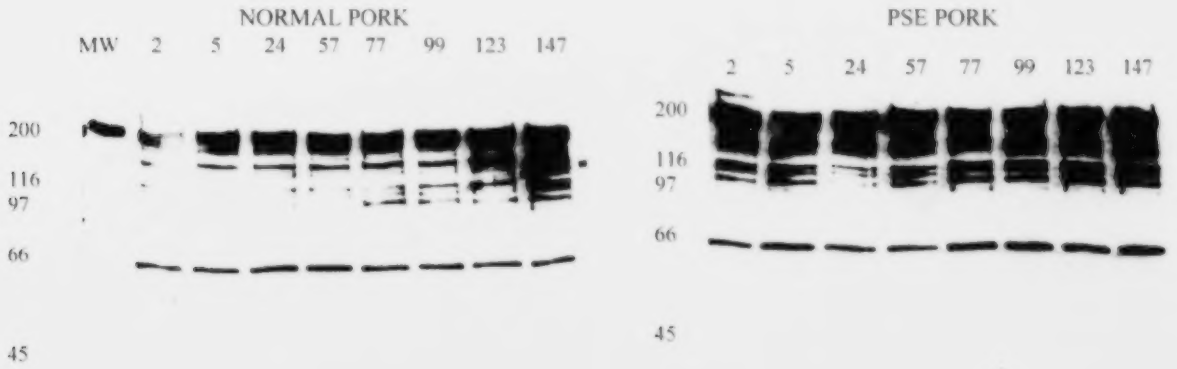

Figure 2. Western blots of pork SDS extracts from Normal and PSE animals. Normal tissue (2003-6) and PSE tissue (2003-10) stored at 4 °C for 2, 5, 24, 57, 77, 99, 123 and 147 h were extracted with SDS containing buffer. The SDS extracts were applied to 8% SDS-polyacrylamide gels (10 µg protein/lane) and transblotted to nitrocellulose membrane. The blots were probed with myosin heavy chain antibody Ab 8 and detected with SuperSignal West Pico Chemiluminescent substrate. Standard myosin was applied to the first lane of the left blot.

Results from our storage studies of meat and extracted NAM suggest that proteins from PSE pork are more susceptible to denaturation and degradation in fresh meat, and following frozen storage. In addition, the initial meat protein denaturation event was found to be the alteration of the tertiary or/and quaternary conformation of actomyosin resulting in a surface hydrophobicity change, while secondary structure (e.g., α-helical content) remained relatively stable. Minor protein degradation (leading to a disruption of the primary structure of protein) was also observed during frozen storage of meat and refrigerated storage of NAM, especially from PSE meat.

1.B. Study of the effect of denaturation and degradation on texture and water binding capacity.

1.B.1. Water Binding Capacity (WBC).

WBC of normal and PSE meat decreased with increasing storage time. The S0-ANS of NAM could explain 55% and 31% of variance of WBC of normal and PSE pork meat, respectively. It is likely that the decrease in WBC of pork meat was partially due to the increasing exposure of the hydrophobic aromatic amino acid residues to the surface of actomyosin. With more hydrophobic amino acid residues on the protein surface, less water can be bound by meat proteins (Wang et al., 2005).

1.B.2. Texture.

The differences in gelation behavior between normal and PSE meat and differences in thermally induced changes in muscle and natural actomyosin (NAM) were examined, in an attempt to better understand how proteins are affected by the PSE condition. It is well known that PSE tissues make inferior meat products, especially for gel products and ham (Kauffman and others 1978; Honkavaara 1988; Honkavaara, 1989; Frisby *et al.*, 2005). However, there were no studies comparing the thermal denaturation and gelation properties from PSE and normal pork using the same animals. Thermal denaturation properties of PSE and normal pork muscle were studied by differential scanning calorimetry (DSC). Gelation properties of low-fat meat gels made from normal and PSE pork loin were also evaluated. Meat gels containing 14% protein, 1.14% crude fat, 81.5% moisture, 2.0% salt and 0.4% sodium tripolyphosphate were prepared from normal and PSE meat (total of 15 animals) using commercial meat processing equipment. The meat gels from PSE pork had lower shear stress ($p < 0.01$) and shear strain ($p < 0.001$) as measured by torsional gelometry, and lower hardness ($p < 0.01$) and chewiness ($p < 0.05$) as measured by Texture Profile Analysis (TPA) than comparable meat gels from normal quality pork (Table 1).

Table 1. Proximate analysis of the pork meat gels and pH, cooking loss, expressible moisture, purge and textural properties of meat gels from two quality groups.

	Normal (n = 8)		PSE (n = 7)		
Measurements	mean	SEM[a]	mean	SEM	p^b
Moisture %	81.6	0.11	81.4	0.21	0.395
Protein %	13.8	0.12	13.9	0.26	0.742
Fat %	1.14	0.14	1.14	0.10	0.973
Meat slurry pH (raw)	5.45	0.06	5.29	0.06	0.102
Meat gel pH (after cooking)	5.91	0.03	5.82	0.03	0.040*
Cooking loss %	0.27	0.04	1.97	0.69	0.049*
Expressible moisture %	36.32	1.17	42.13	1.51	0.011*
Purge %	5.30	0.33	6.22	0.22	0.040*

Texture Profile Analysis (TPA)					
Hardness N	89.56	9.18	57.45	1.10	0.009**
Cohesiveness	0.284	0.01	0.248	0.01	0.067
Springiness cm	1.07	0.02	1.12	0.02	0.126
Chewiness N×cm	29.00	5.05	16.01	0.51	0.037*
Torsional Gelometry					
Shear stress, kPa	50.25	3.72	35.84	1.50	0.006**
Shear strain	1.58	0.04	1.22	0.02	0.000***
G at failure, kPa	31.59	1.60	29.44	1.10	0.290
Slope ratio	0.91	0.01	0.96	0.01	0.000***

The texture map (plot of shear stress at failure against shear strain) clearly shows that meat gels from PSE pork loins had both low shear stress at failure ($\leq$ 40 kPa) and strain ($\leq$ 1.3) (Figure 3). This information will be useful for meat scientists for understanding structural properties of meat gels. There were very high positive correlations ($p < 0.001$) between hardness, shear stress and shear strain. This result was in agreement with Montejano *et al.* (1985). Meat gels from PSE meat also had higher cooking loss, expressible moisture and purge ($p < 0.05$) than that from normal meat.

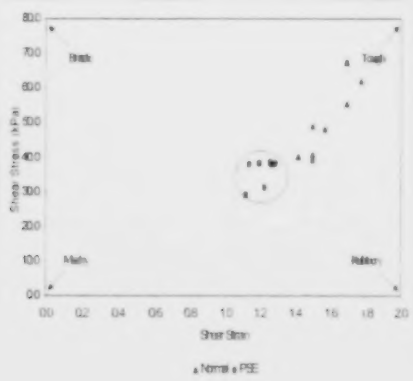

Figure 3. Texture map of meat gels from normal and PSE pork.

Another difference between normal and PSE pork was observed from DSC analysis of pork meat, which resulted in three clearly defined endothermic peaks. The thermal stability (denaturation temperature and enthalpy) of peak 1 of PSE meat was significant lower than that from normal meat. The denaturation temperature of peak 1 was 53.74 and 52.57 °C for normal and PSE meat, respectively, while denaturation enthalpy of peak 1 was 0.93 and 0.67 J/g for normal and PSE meat. In addition, denaturation enthalpies of peak 2 ($p < 0.05$) and total enthalpy ($p < 0.01$) of

PSE meat were also significantly lower than that observed for normal meat. Peak 1 is thought to represent events related to myosin. The new denaturation temperature and denaturation enthalpy data from normal and PSE pork suggest that the inferior quality of meat gels prepared from PSE meat may have resulted from the lower protein thermal stability, particularly of myosin, in PSE meat.

Objective 2: to identify if proteolysis by specific proteases (or other mechanisms) is responsible for degradation and impairment of gelation.

In order to identify the enzyme(s) that degrade myosin in PSE pork and on storage of normal meat, the digestion patterns of myosin by calpain, cathepsin B and cathepsin D were generated and compared with those of the PSE samples. Determination of time courses of digestion of purified myosin by these proteases were carried out. Both calpain and cathepsin D digested myosin at a rapid rate but produced different digestion patterns. On the other hand, pork myosin is not a good substrate for the cathepsin B used in this study. Myosin was digested at a slow rate in spite of the high concentration of the protease used in this study.

Comparison of the digestion patterns of myosin by the different proteases with the PSE profile suggested that the latter was generated by proteolysis of myosin by more than one enzyme. Some of the bands in the PSE sample could be correlated to MHC digestion products by calpain and others by cathepsin D. However, there are other bands in the PSE sample that do not correspond to the digestion products of either protease. These observations suggest that MHC in PSE pork is degraded by more than one protease, probably calpain, cathepsin D and other unidentified proteases.

To further study the proteolysis of myosin, normal and PSE pork were minced, incubated at different pHs (3.4, 5.0, 7.0 and 8.0) for 74 hours at 4 °C in the presence and absence of exogenous proteases. Cathepsin D was added to the sample at pH 3.4, calpain to pH 7.0, and both cathepsin D and calpain at pH 8.0 (Figure 4). It was observed that at pH 3.4, significant amount of digestion occurred after incubation for 74 hours in normal and PSE meat. Addition of cathepsin D to these samples further degraded MHC, yielding a pattern that is very different from that of the sample incubated for 74 hours. Degradation also occurred at pH 7.0 but to a lesser extent than that at pH 3.4. Addition of calpain to the samples further degraded MHC in the PSE sample. At pH 8.0, very little degradation was observed due to prolonged incubation at 4.0 °C. Furthermore, addition of calpain and cathepsin D to the meat at pH 8.0 did not result in significant further degradation. These results suggest that the degradation of MHC by the proteases could be inhibited significantly by increasing the pH of the meat to alkaline pH.

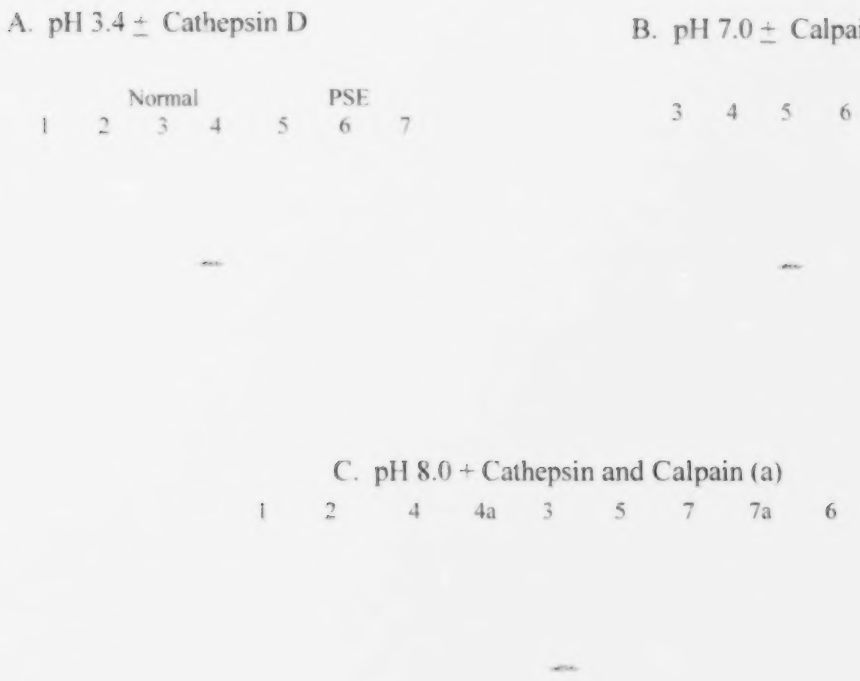

Figure 4. Western blots of WMF from normal and PSE pork incubated at different pH in the presence and absence of proteases. Minced normal (lanes 2-4) and PSE (lanes 5-7) meat were incubated at pH 3.4 (A), 7.0 (B) and 8.0 (C) at 4 °C for 74 hours in the absence (lanes 3 and 5) and presence of cathepsin (lanes A4, A7, C4 and C7) or calpain (B4, B7, C4a and C7a). Myosin standard (BioRad) was applied in lane 1. Pork WMF prepared from minced meat without incubation was applied to lane 2.

Objective 3. to evaluate the effect of fiber type on development of PSE and on protein degradation.

Mammalian muscles consist of a mixture of four primary fiber types; 1, 2a, 2x and 2b. Each of these fiber types has a characteristic physiology and biochemistry. Previous studies have shown that those muscles consisting primarily of type 2b (also termed fast-twitch glycolytic) fibers are predisposed to the PSE condition (Greaser, Okochi & Sosnicki, 2001). Type 2b fibers are the fastest and most powerful contracting fiber type. They are also fast-fatiguing. Type 2b fibers are anaerobic, and metabolize glycogen to provide the energy to power contraction. The biochemical breakdown of glycogen produces lactic acid, which lowers intracellular pH thereby inhibiting muscle contraction. Postmortem, glycogen is also reduced to lactic acid. A rapid reduction in pH in early postmortem muscle is thought to initiate the PSE condition.

In this study we further examined the role of fiber type in PSE muscle. The pork longissimus muscle contains mainly type 2b fibers. We determined whether there was an increased occurrence or size of type 2b fibers, or if type 2b fibers accounted for a greater percentage of the total area of muscle fibers in PSE muscle compared to Control longissimus.

Muscle was obtained from Mitchell's Gourmet Foods Inc, in Saskatoon. Pigs numbered 1, 9, 12, 13, 14, 16 from 2002 were classified as Control, as were pigs numbered 3,6,7,11,12 from 2003. Pigs numbered 6, 7, 8, 10, 11 and 18 from 2002 were classified as PSE, as were pigs numbered 8,9,10,17,20 from 2003. Tissue samples were obtained 56 hours postmortem from each muscle, and were taken from the superficial lateral posterior end of the longissimus muscle. The samples were then frozen in 2-methylbutane cooled by liquid nitrogen and stored at -80°C until use.

The recommended approach of Lefuacheur et al. (2002) was used in selecting the appropriate antibodies, in order to accurately classify pork muscle fiber types.

Antibody*	Binds to	Working Dilution
MHCs	slow twitch fibers (type 1)	1:40 dilution
MHCf	fast twitch fibers (type 2a, 2x, 2b)	1:40 dilution
S5-7D4	type 2a and 2x fibers	1:5 dilution
S5-8H2	type 1, 2x, and 2b fibers	1:5 dilution

**MHCs and MHCf are mouse monoclonal Abs from Novocastra (Newcastle, UK) and S5-7D4 & S5-8H2 are mouse monoclonal Abs from Biocytex (Marseille, France).*

Serial transverse sections were cut at –20°C, using a cryostat, and labeled using immunocytochemical techniques and monoclonal antibodies (MHCs, MHCf, S5-7D4, S5-8H2)

specific for myosin heavy chains (MHC) isoform(s). On this basis fibers were classified as type 1, 2a, 2x or 2b. Immunocytochemical methodology followed our earlier work (Rosser et al., 2000). In addition, to assist in confirming the identity of various fiber types, the fibers were stained for Nicotinamide Adenine Dinucleotide (NADH) activity. The method followed was that of Dubowitz (1985).

Images were captured using a Zeiss photomicroscope, a Sony digital camera, and a Macintosh computer. Next NIH Image (developed at the US National Institutes of Health and available from the Internet by anonymous FTP from *zippy.nimh.nih.gov*) was used to analyze the images by measuring the ellipse minor axis and cell area.

Statistical analyses were performed using SYSTAT (2000). Analysis of variance (ANOVA) was used to detect differences in size, percent or total area of fiber types between normal (control) and PSE pork longissimus muscle tissue. The model used included Tissue Type (control or PSE), Fiber Type (1, 2a, 2b, and 2x) in a two-way factor analysis with an interaction term.

Fibers were classified as type 1, 2a, 2x or 2b. A few rare fibers showed characteristics of both type 1 and type 2 and, consequently, were designated type 1/2. Due to their extreme scarcity, these very few type 1/2 fibers were not include in statistical analyses.

3. A. 1. Percentage of all Fibers

This study did not find a significant difference ($p > 0.5$) in the percentage of type 1, 2a, 2x or 2b fibers between PSE and Control pork longissimus muscle. In both Control and PSE animals, just over one-half of the fibers are type 2b (Table 2).

Table 2. Summary of Fiber Type Percent Data.

	Percentage of all fibers: Control N=11animals			**Percentage of all fibers: PSE** N=11animals		
Fiber Type	**Range (%) Min. – Max.**	**Mean % of fibers**	**Standard Error**	**Range (%) Min. – Max.**	**Mean % of fibers**	**Standard Error**
1	8.4 - 17.81	13.79	0.88	6.34- 24.32	12.09	1.59
2a	3.78 – 12.98	9.29	0.94	3.16 - 14.79	8.72	0.96
2x	13.35 -39.19	25.26	2.81	10.43-33.73	23.55	2.12
2b	30.28-67.35	51.62	3.39	38.64-65.83	55.10	2.80
1\2	0.00 – 0.26	0.04	0.012	0.00-2.66	0.54	0.34

Each fiber type is expressed as a percentage of the total fiber population.

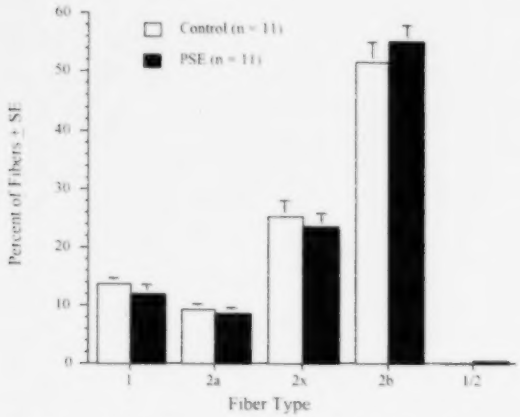

Figure 4. Plot of Fiber Type Percentages. Data are expressed as Mean ± SE.

The percent of the total fiber population comprised by each fiber was plotted as shown in Figure 4. For each fiber type, there is no statistical difference ($p > 0.05$) between Control and PSE animals.

3.A.2. Fiber Size

The relative size of each fiber type is demonstrated by mean ellipse minor axis (fiber diameter). In both PSE and Control animals, type 2b are the largest fibers (Table 3). Overall, fibers from PSE animals were significantly ($p = 0.031$) smaller than fibers from control animals.

Table 3. Summary of Fiber Type Size Data.

	Ellipse minor axis: Control N=11animals			**Ellipse minor axis: PSE** N=11animals		
Fiber Type	**Range (microns)**	**Mean ellipse minor axis**	**Standard error**	**Range (microns)**	**Mean ellipse minor axis**	**Standard error**
1	47.87 - 60.62	52.53	1.42	44.90 – 57.95	49.79	1.35
2a	40.65 – 50.57	46.17	0.96	39.09 – 53.20	44.26	1.23
2x	49.58 - 76.41	58.97	2.65	42.45 – 70.31	54.73	2.76
2b	55.88-73.62	63.64	1.78	49.37 – 67.10	60.93	1.96
1\2	27.82	27.82		39.51 – 47.40	43.31	1.19

The size (as measured by ellipse minor axis) of each fiber type in both Control and PSE animals was also plotted (Figure 5). Excepting the rare hybrid (type 1/2) fibers, in each instance the fibers are smaller in PSE animals. Overall, fibers from PSE animals are significantly smaller ($p>0.031$)

than are those of Control animals.

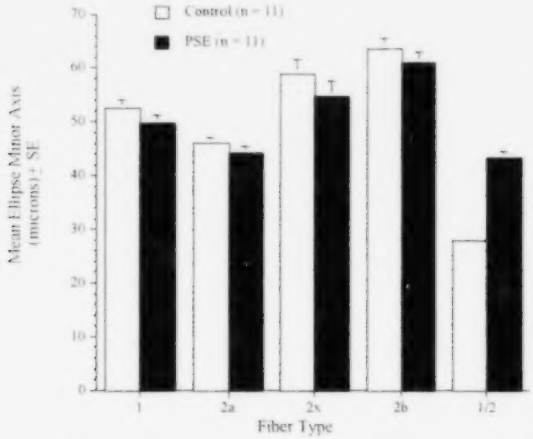

Figure 5. Plot of Fiber Type Size. Data are expressed as Mean ± SE.

3.A3. Percent of total area

The percent of the total cross section area occupied by each fiber type in Control and PSE animals was calculated. In both Control and PSE animals, type 2b fibers occupy over one-half of the cross-sectional area of the muscle (Table 4). There was a no significant difference ($p > 0.5$) in the percent of total cross-sectional area occupied by type 1, 2a, 2x or 2b fibers between PSE and Control pork longissimus muscle.

Table 6. Summary of Fiber Type Total Area Data. (n=11 animals in each group)

	Control			PSE		
Fiber Type	**Range (%) Min. – Max.**	**Mean % of total area**	**Standard Error**	**Range (%)Min. – Max.**	**Mean % of total area**	**Standard error**
1	6.25 - 14.76	10.72	0.86	4.61 - 13.64	8.94	0.77
2a	1.75 - 9.25	5.66	0.68	2.12 – 9.99	5.32	0.67
2x	10.91 - 42.56	25.27	3.55	6.12 - 37.40	22.62	2.77
2b	33.43 – 75.73	58.34	4.10	44.81 – 77.57	62.85	3.09
1\2	0.00 – 0.05	0.01		0.00 – 1.57	0.26	

The percent of the total cross section area occupied by each fiber type in Control and PSE animals is plotted in Figure 6. The differences between Control and PSE animals in the area occupied by each fiber type is not significantly ($p>0.05$) different.

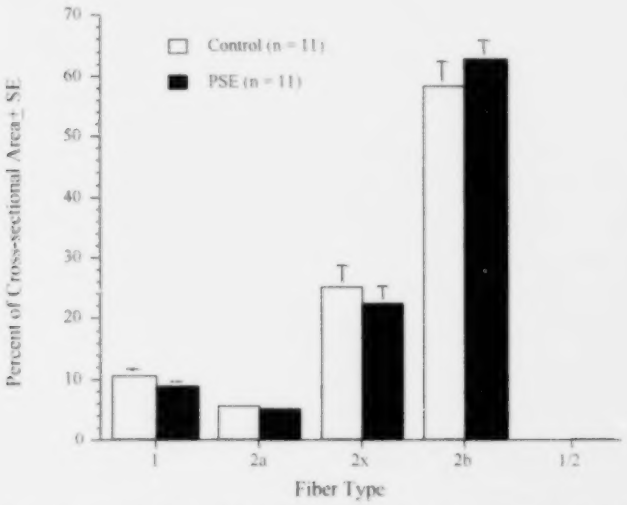

Figure 6. Plot of Fiber Type Total Area. Data are expressed as Mean ± SE.

Type 2b fibers comprise just over one-half of the fiber population and roughly 60% of the total cross-sectional area of the pig longissimus muscle. However, we did not find any differences between PSE and Control muscle in either relative percentage of each fiber types nor total percentage of cross-sectional area of the muscle occupied by each fiber type. These findings demonstrate neither that the relative abundance of type 2b fibers alone, nor that of any other fiber type, is wholly responsible for the occurrence of PSE in pork longissimus muscle.

Overall fibers in PSE muscle were significantly smaller than those of Control muscle. This might have reflected shrinkage due to drip loss from the PSE fibers. The finding of smaller fibers in PSE muscle attests to the validity of the criteria that were used to classify muscle as either PSE or Control. The lack of differences in fiber types between PSE and control animals indicates that factors other than fiber type alone must be responsible for the acquisition of the PSE condition. The smaller fiber size in PSE animals is consistent with loss of fluid from the muscles. Further work in this area should involve the evaluation of isoforms of the various fiber types.

Objective 4. to develop control strategies to limit gelation impairment in PSE muscle.

As discussed in Objective 1 (1.B.2), study of the gelation behavior of normal and PSE revealed that the meat gels from PSE pork have lower shear stress and shear strain compared to meat gels from normal meat. The inferior quality of meat gel prepared form PSE meat may be due to lower protein thermal stability of myosin.

In order to understand the gelation mechanism in meat proteins from normal and PSE pork and to answer the question of why PSE pork makes inferior meat products, we focused on the NAM model system. Ziegler and Acton (1984), Asghar *et al.* (1985) and Ziegler and Foegeding (1990) have described three-step (denaturation, unfolding and aggregation) protein gelation mechanisms in their reviews. Based on the present comprehensive study, including denaturation temperatures by DSC and temperatures at Vmax of S_0-ANS, α-helical content and turbidity), NAM gelation is postulated to include five steps as follows: (1) Energy absorption. NAM absorbs the energy provided by heating which increases molecular movement. (2) Conformational change. The energy is absorbed by protein, resulting in NAM conformational (tertiary or/and quaternary structure) change as indicated by S_0-ANS. (3) Unfolding. With more energy absorbed, the NAM underwent a secondary structure change as indicated by the reduction in α-helical content. (4) Aggregation. The unfolded peptides become linked by hydrophobic interactions and disulfide bonding. The aggregation may also have involved electrostatic and hydrogen bonding and van der Waals interactions. (5) Finalized network. The proteins continuously absorb the energy provided to finalize the chemical bonding.

It must be noted that the absorbed energy is the main driving force for protein gelation, and quality and quantity of chemical bonding is the key for protein network development. It seems logical that the protein structural changes follow these five steps in a sequential order based on the temperatures at Vmax of each parameter, although considerable overlap exists.

This work also provided new evidence on the important roles of hydrophobic interaction, disulfide bonding and unfolding in thermal gelation of muscle proteins. The S_0-ANS value of NAM at 80 °C can explain about 65%, 50% and 48% of variance of shear stress, hardness and shear strain of meat gel, respectively. At 80 °C, about 44% and 26% of sulfhydryl groups formed disulfide bonds or became covalently linked to other amino acid residues in NAM from normal and PSE pork, respectively. About 90% of the alpha–helical structure of the actomyosin molecules were unfolded by heating to 70 °C or higher. The alpha–helical content decline (unfolding) in NAM related well to the thermal transitions revealed by DSC analysis.

We did not completely achieve our goal of developing control strategies to limit gelation

impairment in PSE meat. First there was much that we still do not understand about the gelation process itself in PSE meat, so we chose to expend more of our efforts in that direction. Also, since one specific protease was not the primary cause of degradation in PSE meat (as observed under objective 2), we did not attempt gelation studies with added protein inhibitors. We did observe that hydrophobicity increased with frozen storage, and so it is recommended that PSE meat should not be stored as a frozen product for long periods of time before further processing or value-addition, as the resulting products will have lower water holding capacity and impaired texture.

The thermally-induced gelation of meat protein is a series of protein structural changes and chemical interaction (bonding) of the proteins with the supply of energy. The comprehensive studies of thermally-induced gelation of NAM from normal and PSE pork allowed us to postulate a more detailed mechanism of meat protein gelation. The different thermally-induced gelation behavior of NAM extracted from normal and PSE pork was attributed to the denaturation status of the original proteins. NAM from normal pork underwent aggregation through a higher extent of hydrophobic interaction and disulfide bonds, as well as higher temperature in folding and maximum S_0-ANS change than that from PSE pork. As a consequence, NAM from normal pork had superior rheological properties.

NEXT STEPS

This work provided new insight into how the structure of meat proteins is changed (denaturation and degradation) during refrigerated storage, frozen storage and thermally-induced gelation. Therefore, in addition to the consideration given for genetic improvement of meat quality, preventing meat protein denaturation, in particular, immediately after pork slaughter should be an important consideration for the meat industry.

A detailed evaluation of early postmortem intervention strategies such as rapid chilling was beyond the scope of the present study, but should be considered in the future. This would involve control and manipulation of events and procedures both before and after slaughter, which is not possible in a commercial slaughter facility such as Mitchell's. We are currently investigating collaborative studies with Agriculture and Agri-Food Canada in Lacombe Alberta as they have research slaughter facilities that we might be able to access. We are also presenting our findings to industry and hope to gain their cooperation in continuing this work.

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STATEMENT OF EXPENDITURES

A statement of expenditures prepared by Research Accounts, University of Saskatchewan has been forwarded.

APPENDIX 1: Summary of Methods Used

Sample Selection: Pork carcasses were initially selected from a commercial pork processing line based on visual assessment of color, onset of rigor and the pH at 45 min post mortem (pH_{45}). The pH_{45} was obtained 45 min after exsanguination, just before the carcasses entered the blast chiller, using two HI 9025 portable microcomputer pH meters (HANNA Instruments, Ann Arbor, MI, U.S.A.) with spear-tipped electrodes. The probe was inserted into the *longissimus dorsi* muscle at a point near the 10th rib and rinsed with deionized water between readings. Carcasses with a pH_{45}<5.8 (as suspect PSE samples) and pH_{45}> 6.0 (as suspect normal samples) were selected. At 2 and 5 h post mortem, loin core samples (approximately 10 g) near the 9th rib were taken and frozen in liquid nitrogen for determination of iodoacetate pH. A section of the loin was removed from the right side of each carcass at 24 h post mortem, kept in an insulated cooler and transported to the University of Saskatchewan. A denuded loin sample (approximately 1.4 kg) was obtained by removing the skin and trimming the visible fat and connective tissues in a refrigerated pilot plant (< 7 °C) in the College of Agriculture.

Determination of pH_2, pH_5 and pHu: Iodoacetate pH of meat samples was measured in duplicate according to Jeacocke (1977). Frozen 2 g core samples taken at 2 and 5 h post mortem were homogenized with a Polytron (Type PT10/35, Kinemetica GmbH, Switzerland) on #6 setting for 30 s at 4 °C in 18 mL of 5 mM sodium iodoacetate (to arrest glycolysis), 150 mM KCl, pH 7.0. The pH of the homogenate was then measured at room temperature with a temperature calibrated pH meter with an Accumet pH electrode (Fisher Scientific, Nepean, ON, Canada) and referred to as pH_2 and pH_5. The iodoacetate pH of fresh meat at 30 h post mortem was determined as described above and referred to as ultimate pH (pH_u).

Determination of Muscle Slurry pH: Muscle slurry pH of fresh (72 h post mortem) and thawed meat after frozen storage was determined as described above, except that deionized water was used.

Drip Loss: The drip loss was measured in duplicate according to Warner and others (1997) with some modifications. A standard size sample (3.5 cm thick, 4 cm length and 4 cm width chop adjacent to 12-13th costae, approximately 50 g) at 26 h post mortem was removed and placed on a Styrofoam tray with a soaker pad inside a polyethylene bag. The meat was weighed before and after 48 h storage at 4°C. The drip loss is expressed as the percentage weight loss over 48 h.

Colour: Objective colour readings were determined in duplicate on a freshly cut loin surface adjacent to the 11th costae at 26 h post mortem. The 2 cm thick chop was placed on a Styrofoam tray and over-wrapped with oxygen permeable film (30000 cm^3/m^2/24 h; Vitafilm, Huntsman Film Products of Canada, Toronto, ON, Canada). After a 30 min bloom at 4°C, the colour was determined using a Hunter Lab Miniscan XE (HunterLab 1149, Reston, VA, U.S.A.) colorimeter with a 1 inch port, which was set at L^*, a^*, and b^*, observer 10 and illuminant A. The instrument was previously standardized using black and white tiles. The color of a pink tile was measured each day during testing to assess the instrument performance. After taking the first reading, the sample was rotated clockwise 90° and a second measurement was made. These measurements were used to confirm the assignment of samples to one of two quality classes (normal or PSE) based on the following parameters.

1. Normal: pH_{45} > 6.0, pH_2 > 6.0, pH_u <5.8, L^* 44-50, drip loss < 3%
2. PSE: pH_{45} <5.8, pH_2 <5.8, pH_u <5.8, L^* >50, drip loss > 3%

Frozen Sample Preparation: Four PSE and four normal pork loins were selected for further study. To minimize variability due to muscle location, an 18 cm section from the posterior half of the loin was cut into about 2 cm cubes, mixed, packed (about 80 g per bag) in sterile sample bags (VWR Scientific Products, Mississauga, ON, Canada). One sample from each loin was analyzed fresh at 72 h post mortem, and the remaining samples were frozen at –30 °C for 16 h and then stored at –20 °C. These samples were assessed following 1, 2, 4 and 6 mo of frozen storage.

Thaw Loss: Thaw loss was determined according to Wynveen and others (2001) with some modifications. At each time interval, frozen loin samples were thawed at 4 °C for 16 h, removed from the sample package, blotted dry, and reweighed. Thaw loss was expressed as the percentage difference in weight from the initial frozen sample.

Sample Preparation For NAM Extraction, Slurry Ph And Water-Binding Capacity: At each time interval, one subsample (80 g) from each of 4 normal and 4 PSE pork loins was ground for 30 s using an Osterizer Model 548 Blender (Sunbeam Corp., Toronto, ON, Canada). The ground samples were used for determination of slurry pH and WBC and preparation of NAM.

Water-binding Capacity Of Pork Muscle: WBC was determined in triplicate as described by Wang and others (2003). Briefly, about 2.5 g of ground pork muscle was mixed well with 5 mL of cold deionized water in a 50 mL centrifuge tube. The mixture was allowed to stand for 24 h at 4°C. After centrifugation at 1800 × g for 10 min at 4 °C (J2-HC, Beckman Instruments, Inc., CA, U.S.A.), the pellet was weighed. WBC was expressed as a percentage of the gain in weight of the pellet.

Extraction of Pork Natural Actomyosin: NAM was extracted according to Kim and others (1993) and Ogawa and others (1999) with slight modifications. Twenty grams of ground pork in 100 mL of 50 mM KCl, 20 mM potassium phosphate buffer, pH 7.0 was homogenized for 15 s by using an Osterizer Blender. The homogenate was centrifuged at 10000 × *g* for 5 min at 4°C using a Beckman J2-HC centrifuge. The homogenization and centrifugation of the sample were repeated. The pellet was then homogenized for 15 s with 200 mL of 0.6 M KCl, 20 mM potassium phosphate buffer, pH 7.0. The homogenate was centrifuged at 10000 × g for 5 min. The supernatant was filtered through three layers of cheesecloth, grade 50 (Veratec Graphic Arts Cheesecloth, Simpsonville, SC, U.S.A.). The filtered solution was diluted with 1200 mL of 20 mM potassium phosphate buffer, pH 7.0 and stirred for 30 min. Following centrifugation at 10000 × *g* for 10 min, the pellet was resuspended in 50 mL of 50 mM KCl, 20 mM potassium phosphate buffer, pH 7.0. The washed precipitate was collected by centrifugation at 10000 × *g* for 10 min. All procedures were carried out at 4 °C. The pellet from the final centrifugation step is hereafter referred to as "natural actomyosin" or NAM.

Differential Scanning Calorimetry (DSC): DSC was performed on a TA Modulated DSC thermal analyzer (TA Instruments, New Castle, DE, U.S.A). Each pork sample was thawed at 4 °C for 16 h before DSC analysis and then finely minced in a food processor. Meat from seven PSE and five normal animals (15-25 mg) was accurately weighed into the aluminum pan. The pan was hermetically sealed and then heated from 20 °C to 90 °C at a rate of 5 °C/min. A sealed empty pan was used as a reference. Denaturation temperature (Td, also called peak transition temperature or Tm), and enthalpy of denaturation (ΔH) were computed from the thermograms by a Universal Analysis Program, Version 3.9A (TA Instruments, New Castle, DE, U.S.A). Temperature calibration was done with indium. Each sample was analyzed in duplicate.

Preparation of Low-Fat Meat Gels: Pork loins from seven PSE and eight normal animals were used. From each animal, 4-kg batches of each treatment were prepared. In order to minimize the effect of ingredients, only four specific ingredients were used, namely meat, ice water, sodium chloride and sodium tripolyphosphate. Formulations consisted of 60.0% pork, 37.6% ice water, 2.0% sodium chloride (Windsor, ON, Canada), and 0.4% sodium tripolyphosphate (UFL Foods Inc., Toronto, ON, Canada). This resulted in meat gels containing 14% protein, 1.14% crude fat and 81.5% moisture. Processing was carried out in a refrigerated pilot plant as described by Shand (2000) with modifications. Pork loins were thawed at 4 °C for 48 h before processing and then ground through a 3 mm plate (model AMFG-24, Biro Grinder, Marblehead, OH, U.S.A) twice. Initial processing for all treatments involved mixing (model Berkel, Ba-20, South Bend, IN, U.S.A) the dry ingredients with the meat during the first min. Ice water (0-1 °C) was then added and the batter mixed for 3 min. Treatments were processed through an emulsion mill (Type 1E-75-F, Alexanderwerk, Remscheid, Germany). Temperatures of meat batters following emulsification were similar (3.5 to 5.5 °C). Each batter was then placed in a vacuum bag and a vacuum (-80 kPa) was drawn three times to remove as much air as possible. The batter was then stuffed into moisture-proof casings using a Handtmann VF80 stuffer (Biberach /Riss, Germany). Products were then tensioned by hand and clipped. Stuffed chubs were held overnight (4

°C), and then cooked to 74 °C using a five stage cooking process and temperature controlled as described by Shand (2000). Products were then water-cooled to 35 °C (30 to 40 min), chilled overnight at 4 °C and reweighed. Three chubs per treatment were opened for cook yield determinations. The remainder was stored at 4 °C for texture evaluation and chemical analysis.

Cooking Losses: Cooking losses was measured according to Shand (2000) with some modifications. Three chubs per treatment were opened, blotted and the weight of meat gel and peeled casing was determined. Cook losses was calculated based on the raw product weight.

Purge From Cooked Gels: Purge was measured according to Shand (2000) with some modifications. Purge accumulation from sliced product was determined in duplicate. Twelve slices of meat gel (3 mm thick) were vacuum-packaged (-90 kPa, Roschermatic Type VM-20, Osnabruck, Germany) in each of two preweighed high-barrier, mylar/polyethylene bags (oxygen permeability was 7.7 mL/m^2/24 h at room temperature). Packages were stored in an upright position for 7 days at 4 °C, and then the slices were blotted and reweighed.

Expressible Moisture: Expressible moisture was determined using a modification of the method described by Jauregui and others (1981) as described by Shand (2000). All samples were run in triplicate and the expressible moisture reported as percent weight lost from the original sample.

Torsional Gelometry: Torsional gelometry was measured according to the method of Rathgeber and others (1999a, 1999b). The cooked gels were removed from the casing, blotted dry, and accurately cut into 28.7 mm long cylinders. Styrene discs were attached to each end of the prepared cores (19 mm in diameter, 15 samples per treatment) using cyanoacrylate glue (Loctite 404, Instant Adhesive, Loctite Corporation, CT, U.S.A). These samples were milled into capstan-shapes with a center diameter of 10 mm using a modified bench grinder (KCI-24A2, Bodine Electric Company, IL, U.S.A). After shaping the samples were placed in a plastic bag (to prevent moisture loss) and brought to room temperature. For testing, each sample was vertically mounted and twisted to fracture at 2.5 rpm on a torsion gelometer (Brookfield digital viscometer model DV-I with the torsion fixture attached, Gel Consultants Inc., Raleigh, NC, U.S.A). The shear stress and true shear strain at fracture was determined.

Texture Profile Analysis (TPA): TPA was performed according to the method of Shand (2000) with some modifications. Eight cores (2.2 cm diameter, 1.5 cm high) was cut from the center of each chub and allowed to equilibrate to room temperature. Each core was axially compressed 75% of its height for 2 cycles (crosshead speed 0.167 cm/s) on a Food Technology Corp. TMS-TP Texture Press (Food Technology Corp., Rockville, MD, U.S.A). TPA values were calculated using the supplied software as follows: hardness (peak force [N] required for first compression); cohesiveness (ratio of the active work done under the second compression curve to that done under the first compression curve [dimensionless]); springiness (distance [cm] the sample recovers after the first compression) and chewiness (hardness × cohesiveness × springiness [N×cm]).

Meat Slurry pH, Meat Gel pH And Proximate Chemical Composition Analysis: Meat slurry pH (thawed meat at time of gel preparation) and pH of cooked meat gels were determined as described for pHu, except that deionized water was used. The proximate chemical composition, that is, moisture was determined by measuring weight lost after drying in a 105 °C oven for 16-18 h (950.46, AOAC, 1990) and protein was calculated from total Kjeldahl nitrogen determination (981.10, AOAC, 1990) using 6.25 as the conversion factor. The crude fat content was determined using petroleum ether as the solvent (960.39, AOAC, 1990). All samples were analyzed in duplicate.

Protein Concentration Assay And Extractability Of NAM: The protein content was determined according to the BCA (bicinchoninic acid) protein assay procedure provided by PIERCE (Dartmouth NS, Canada). Bovine serum albumin (BSA) provided in the PIERCE BCA protein assay reagent kit was used as the standard. Measurements were done in duplicate. Extractability of NAM was calculated and expressed as milligrams of NAM protein per gram of muscle.

Heat Treatment of NAM: About 10 mL of NAM solution (5.0 mg protein/mL in 0.6 M KCl, 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0) from each sample was poured into 9 sterile Falcon centrifuge tubes and covered with lids to avoid dehydration during heating in a water bath. The sample temperatures were raised at a constant rate of 1.3 °C min^{-1} from 22 to 90 °C. The temperature at the center of the tube was monitored using a copper-constantan thermocouple. The NAM solution was immediately cooled in ice-water once the central temperature of samples reached 25, 40, 50, 55, 60, 70, 80 or 90 °C. An unheated solution kept at 4 °C served as a control.

Determination of α–Helical Content of NAM: The α–helical content of thermally treated NAM was measured according to Ogawa et al. (1999) with some modifications. NAM was diluted to 0.2 mg/mL with 0.6 M KCl, 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0. Circular dichroism (CD) spectra and ellipticities at 222 nm were obtained with a π*-180 Circular Dichroism spectrometer (Applied Photophysics Ltd, UK) in a 1 mm path length quartz cell. A water-jacketed cell holder was used to maintain a constant temperature by circulating water at 4°C. The instrument was calibrated by using d-10-camphorsulfonic acid ($[\theta]_{290.5}$ = 7800 deg.cm^2.$dmol^{-1}$) before each measurement. The α–helical content was estimated as described by Wang et al. (2005).

Differential Scanning Calorimetry (DSC) of NAM: DSC analysis of NAM (20 mg/mL in 0.6 M KCl, 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0) from normal or PSE meat was performed on a TA Modulated DSC thermal analyzer (TA Instruments, New Castle, DE, USA). Each NAM solution (15-25 mg) was accurately weighed into aluminum pans which were hermetically sealed and then heated from 20 °C to 90 °C at a rate of 5 °C/min. A sealed pan containing 15 mg of the same buffer was used as a reference. Denaturation temperature (Td, also called peak transition temperature), and enthalpy of denaturation (ΔH) were computed from the thermograms using a Universal Analysis Program, Version 3.9A (TA Instruments, New Castle, DE, U.S.A). Temperature calibration was done with indium as a standard. Each sample was analyzed in duplicate.

Measurement of Surface Hydrophobicity of NAM: Protein surface hydrophobicity was determined with the fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (ANS) according to Li-Chan et al. (1985, 1986) as described by Wang et al. (2005). All fluorescence measurements were made with a SPEX Fluorolog 1680 double spectrometer (Jobin Yvon Inc., Edison, NJ, U.S.A.) with the excitation and emission wavelengths at 390 and 470 nm, respectively. The excitation and emission slit widths were 1 nm. The initial slope (S_0) of the plot of the fluorescent intensity against % protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity (S_0-ANS). To correct for instrumental fluctuations in fluorescence intensity, standardization was performed by measuring the fluorescent intensity of 10 μL ANS in 4 mL methanol and corrected to a value of 30.

Measurement of Sulfhydryl and Disulfide Contents in NAM: Total and surface sulfhydryl (S-SH) (also called reactive sulfhydryl) contents of heat-treated NAM were determined in the presence and absence of 8 M urea, respectively, by colorimetric assay using Ellman's reagent 5, 5'-dithio-bis (2-nitrobenzoic acid). The sulfhydryl plus disulfide (SH+SS) content of thermally-treated NAM was measured by using 2-nitro-5-thiosulfobenzoate (NTSB) according to Damodaran (1985) with slight modifications as described by Wang et al. (2005). Disulfide content was calculated as the difference between (SS+SH) and total sulfhydryl contents. Measurements were done in triplicate. The SH and SS contents were calculated from A_{412} values using an extinction coefficient of 13,600 $M^{-1}cm^{-1}$ and expressed as moles SH per 10^5 g protein.

Assessment of Turbidity of NAM: The NAM solution after heat treatment was diluted to 0.5 mg/mL with 0.6 M KCl, 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0. Absorbance at 350 and 660 nm of the diluted protein solution was obtained using a UV/Vis Spectrophotometer (Hewlett Packard 8453, Columbus, OH, USA) and expressed as absorbance of mg/mL of protein.

Dynamic Rheological Measurement of NAM: Dynamic rheological properties of NAM (20 mg/mL in 0.6 M KCl, 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0) from PSE and normal pork were measured by using an AR-1000 advanced rheometer (TA Instruments, New Castle, DE, USA), which was equipped with a parallel plate measuring system (40 mm diameter). The gap between the parallel plates was set at 1 mm. Approximately 1.6 mL sample solution was

applied to the lower plate, and the upper plate was gently lowered under the control of a programmed procedure (AR 1000 Rheometer Solution Software, AR 1000 Module, V1.1.7, New Castle, DE, USA). Light mineal oil was applied to the edge of the parallel plate system to prevent the loss of moisture during heating. The sample was heated at a rate of 1 °C/min from 20 to 80°C, using a programmable temperature-controlled water bath. Data were collected every 30 s and measurements were carried out in duplicate. Oscillatory measurements were made at a fixed frequency of 0.1 Hz and a maximum strain amplitude of 0.01, which was within the linear viscoelastic region as determined in preliminary work (Creep experiments). For all the tests, the storage modulus (G'), loss modulus (G"), tangent plase angle (tan δ = G"/ G') were computed from the raw oscillatory data using software (TA Instruments, New Caste, DE, USA) that accompanied the rheometer.

SDS-PAGE of Thermally Treated NAM: After heat treatment, the supernatant of each NAM sample was mixed with sample loading buffer (10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0002% bromophenolblue, 80 mM Tris-HCl, pH 6.8). The sample was boiled in a water bath for 5 min and stored at -20 °C for later analysis as described by Wang et al. (2005). The SDS-PAGE densitometry profile was scanned using a digital camera (monochrome CCD, RS-170/CCIR camera, Bio-Rad, Segrate, Milan, Italy) and analyzed using Quantity One software (version 4.3.1, Bio-Rad, Mississauga, ON, Canada).

Preparation of Myosin: All procedures were carried out at 4 °C unless specified. The meat (20 g) was ground and homogenized with 80 mL of 0.5% Triton X-100, 50 mM NaCl, 10 mM MOPS (pH 7.0), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF and then centrifuge for 15 min at 9000 rpm. The pellet was washed 4 times with Buffer A (50 mM NaCl, 10 mM MOPS (pH 7.0), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF). The washed pellet was briefly homogenize (3 10-min pulses) in 80 mL extraction buffer, Buffer B (40 mM NaCl, 1mM EDTA, 2 mM EGTA, 40 mM MOPS (pH 7.2), 1 mM DTT, 0.1 mM PMSF, 10 mM ATP in a blender, stirred for 45 min in the cold room and centrifuged at 9000 rpm for 20 min.. The supernatant was made to 0.6 M NaCl, 20 mM MgSO4, 15 mM ATP and subjected to $(NH_4)_2SO_4$ fractionation.. Myosin selectively precipitated in the 42-60% saturation fraction. The pellet was dissolved in the minimum volume of Buffer C (25 mM NaCl, 5 mM MOPS, 0.1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT (pH 7.0) and dialyzed against Buffer C. Equal volume of cold water was added to the dialyzed solution and made to 10 mM $MgCl_2$. The mixture was stirred for 30 min and centrifuged at 11000 rpm for 50 min. The pellet obtained was resuspended in the Buffer A and centrifuge at 11,000 rpm for 15 min. The pellet was homogenized in the washed buffer. The resulting crude myosin was made to 10 mM ATP, 10 mM $MgCl_2$ and purified by gel filtration on a Sepharose 4B column(2.5 x 100 cm) equilibrated with Buffer D (0.5 M NaCl, 15 mM Tris (pH 7.4), 0.1 mM EGTA, 1 mM DTT, 3 mM NaN_3, 0.1 mM PMSF). The fractions were analyzed by SDS-polyacrylamide gel electrophoresis to detect the myosin. The myosin peak was pooled and dialyze against Buffer D overnight with 2 changes. The dialysate was made to 10 mM $MgCl_2$, stir for 1 h in the cold room and centrifuge. The purified myosin pellet was resuspended in the minimum volume of Buffer D and homogenize in a glass homogenizer.

Preparation of Washed Myofibrils: Washed myofibril (WMF) was prepared according to Rathgeber *et al* (1999). Frozen pork meat (250 mg) was crushed on a frozen mortal and pestle and was homogenized in 5 mL of Buffer E (0.05 M potassium phosphate, 1 mM NaN_3, 2 mM EDTA, pH 7.3 for 10 seconds. After 30 minutes in ice, the sample was centrifuged at 17,500 x g for 15 minutes at 4 °C. The supernatant was labeled as LIS (low ionic strength supernatant) fraction . This step was repeated and the pellet obtained following centrifugation was homogenized in 5 mL of Buffer F (0.05 M potassium phosphate, 1 mM NaN3, 2 mM EDTA, 0.55 M KCl, pH 7.3 for 10 seconds. The supernatant obtained after centrifugation of the mixture was labeled as HIS (high ionic strength supernatant) fraction. The pellet was washed 3 times with 5 mM Tris pH 8.0 and labeled as WMF (washed myofibril).

SDS-polyacrylamide Electrophoresis (SDS-PAGE): SDS-PAGE was carried out according to the procedure of Laemmli (1970). The acrylamide gel concentrations used for the resolving gel were 8 – 12.5%. The 12.5% SDS polyacrylamide gels were used to examine the profile of the proteins with molecular weight of 14,000 to 200,000 while the 8% gels were used to analyze the high molecular weight proteins (~30, 000 to 200,000 Da).

Western Blotting Analysis: The meat extracts were applied to 12.5% SDS-polyacrylamide gels and electroblotted to nitrocellulose paper according to a modified method of Towbin et al. (1979). To facilitate the transfer of the proteins to the nitrocellulose paper, 0.1% SDS was included in the transfer buffer. The efficiency of transfer was verified by staining the gel after the transfer with coomassie blue and the nitrocellulose sheet is stained briefly with a-naphthol blue black to visualize the transferred proteins. The position of the molecular weight standards were marked for reference. The blot was blocked with 5% nonfat dry milk in TTBS (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5,). The blots were incubated overnight with the primary myosin antibodies in TTBS with 1% nonfat milk, washed with TTBS and then incubated with horse radish peroxidase conjugated secondary antibodies in TTBS containing 2.5% nonfat milk for 2 hours. The primary antibodies used for detection of myosin are listed below. The cross-reactivity of the antibodies was detected by chemiluminescence using a mixture of equal volumes of enhanced luminol reagent and oxidizing reagent, as recommended by the manufacturer. The blots were exposed to x-ray films for various times, and the films were developed and scanned. The 9 myosin antibodies were used for this study were designated as Ab 1-9 are commercially available. These antibodies are:

Ab1, monoclonal (mouse monoclonal IgG) antibody against rat smooth muscle myosin heavy chain (MHC) (Santa Cruz).
Ab 2, polyclonal Myosin antibodies against human skeletal MHC and light chain developed in rabbit (Sigma).
Ab 3, polyclonal Myosin antibodies against bovine uterus smooth muscle developed in rabbit (Sigma).
Ab 4, monoclonal (mouse IgG1 isotype clone My-32) myosin antibody against rabbit fast skeletal myosin (Sigma).
Ab 5, monoclonal (mouse IgG1 isotype clone 8H2) myosin antibody against slow (isoform I) and fast (isoform II) myosin heavy chain (ACRO).
Ab 6, monoclonal (mouse IgG1 clone 7D4) myosin antibody aginst slow (isoforms I) and fast (isoforms II) myosin heavy chain (ACRO).
Ab 7, monoclonal (clone NOQ7.5.4D) myosin antibody against the heavy meromyosin portion of human adult skeletal muscle slow myosin (Sigma).
Ab 8 – monoclonal (clone NA4) myosin antibody against avian sarcomeric myosins (gift from Dr. E. Bandman, University of California, Davis)
Ab 9, monoclonal (clone AG6) myosin antibody against avian myosin from fast-twitch fibers.

Determination of Fiber Type and Size: Tissue samples were obtained 56 hours postmortem from each muscle, and were taken from the superficial lateral posterior end of the longissimus muscle. The samples were then frozen in 2-methylbutane cooled by liquid nitrogen and stored at -80°C until use. The recommended approach of Lefuacheur et al. (2002) was used in selecting the appropriate antibodies, in order to accurately classify pork muscle fiber types.

Serial transverse sections were cut at –20°C, using a cryostat, and labeled using immunocytochemical techniques and monoclonal antibodies (MHCs, MHCf, S5-7D4, S5-8H2) specific for myosin heavy chains (MHC) isoform(s). On this basis fibers were classified as type 1, 2a, 2x or 2b. Immunocytochemical methodology followed our earlier work (Rosser et al., 2000). In addition, to assist in confirming the identity of various fiber types, the fibers were stained for Nicotinamide Adenine Dinucleotide (NADH) activity. The method followed was that of Dubowitz (1985). Images were captured using a Zeiss photomicroscope, a Sony digital camera, and a Macintosh computer. Next NIH Image (developed at the US National Institutes of Health and available from the Internet by anonymous FTP from *zippy.nimh.nih.gov*) was used to analyze the images by measuring the ellipse minor axis and cell area.

Statistical Analysis: Analysis of variance (ANOVA) was carried out with the level of significance set at $p < 0.05$. Pearson correlations were used for testing correlations among test parameters. All analyses were conducted using Statistical Analysis Program System version 8.2 for Windows (SAS Institute Inc., Cary, NC, U.S.A) or Systat version 10 (SPSS, Inc. 2000).